



## Microorganisms persist at record depths in the subseafloor of the Canterbury Basin.

Maria-Cristina Ciobanu, Gaëtan Burgaud, Alexis Dufresne, Anja Breuker, Vanessa Rédou, Sarah Ben Maamar, Frédéric Gaboyer, Odile Vandenabeele-Trambouze, Julius Sebastian Lipp, Axel Schippers, et al.

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**1 Microorganisms persist at record depths in the subseafloor of the**  
**2 Canterbury Basin**

3

4 MC Ciobanu<sup>1,2,3</sup>, G Burgaud<sup>4,8</sup>, A Dufresne<sup>5,8</sup>, A Breuker<sup>6</sup>, V Rédou<sup>4</sup>, S Ben Maamar<sup>1</sup>, F  
5 Gaboyer<sup>1,2,3</sup>, O Vandenabeele-Trambouze<sup>2,1,3</sup>, JS Lipp<sup>7</sup>, A Schippers<sup>6</sup>, P Vandenkoornhuyse<sup>5</sup>, G  
6 Barbier<sup>4</sup>, M Jebbar<sup>1,2,3</sup>, A Godfroy<sup>3,1,2</sup> & K Alain<sup>2,1,3</sup>.

7

8 <sup>1</sup> Université de Bretagne Occidentale (UBO, UEB), IUEM – UMR 6197, Laboratoire de  
9 Microbiologie des Environnements Extrêmes (LMEE), Plouzané, France

10 <sup>2</sup> CNRS, IUEM – UMR 6197, LMEE, Plouzané, France

11 <sup>3</sup> Ifremer, UMR6197, LMEE, Plouzané, France

12 <sup>4</sup> Université de Brest, UEB, Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne  
13 EA 3882, IFR148 SFR ScInBioS, ESIAB, Plouzané, France.

14 <sup>5</sup> Université de Rennes I, CNRS, UMR 6553 ECOBIO, Rennes, France.

15 <sup>6</sup> Bundesanstalt für Geowissenschaften und Rohstoffe (BGR), Hannover, Germany.

16 <sup>7</sup> Organic Geochemistry Group, Department of Geosciences and MARUM Center for Marine  
17 Environmental Sciences, University of Bremen, Bremen, Germany.

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22

23 Correspondence: K Alain, Laboratoire de Microbiologie des Environnements Extrêmes  
24 (LMEE), UMR 6197 CNRS-UBO-Ifremer, Plouzané, France. E-mail: [Karine.Alain@univ-](mailto:Karine.Alain@univ-brest.fr)  
25 [brest.fr](mailto:Karine.Alain@univ-brest.fr)

26 <sup>8</sup>These authors contributed equally to this work

27 The subsurface realm is colonized by microbial communities to depths of more  
28 than 1000 meters below the seafloor (mbsf), but little is known about overall  
29 diversity and microbial distribution patterns at the most profound depths. Here we  
30 show that not only *Bacteria* and *Archaea*, but also *Eukarya* occur at record depths  
31 in the subseafloor of the Canterbury Basin. Shifts in microbial community  
32 composition along a core of nearly 2 km reflect vertical taxa zonation influenced  
33 by sediment depth. Representatives of some microbial taxa were also cultivated  
34 using methods mimicking *in situ* conditions. These results suggest that diverse  
35 microorganisms persist down to 1922 mbsf in the seafloor of the Canterbury Basin  
36 and extend the previously known depth limits of microbial evidence (i) from 159 to  
37 1740 mbsf for *Eukarya* and (ii) from 518 to 1922 mbsf for *Bacteria*.

38

## 39 **Introduction**

40 In addition to terrestrial and marine near-surface habitats, the deep biosphere is  
41 considered to be a third realm for microbial life. Subseafloor sediments provide a  
42 habitat for large numbers of microbial cells, as revealed by cell counts (Parkes et al.,  
43 2000) or CARD-FISH (Schippers et al., 2005). Although recent data have shown that  
44 the global biomass in subseafloor sediments is smaller than given by earlier estimates,  
45 the deep subseafloor biosphere still constitutes a large fraction ( $2.9 \times 10^{29}$  cells) of  
46 Earth's living biomass (Kallmeyer et al., 2012).

47 The subsurface microbiota is diverse and complex, hosting metabolically active  
48 communities down to depths of more than one thousand meters below the seafloor  
49 (mbsf), as revealed by molecular, metagenomic and metatranscriptomic studies (Lipp *et*  
50 *al.*, 2008; Roussel *et al.*, 2008; Biddle *et al.*, 2011; Pawlowski *et al.*, 2011; Orsi *et al.*

2013a). It harbors representatives from the three domains of life, i.e., numerous endemic and/or as yet uncultured *Archaea* and *Bacteria* (e.g., Orcutt *et al.*, 2011; Inagaki *et al.* 2006), in addition to bacterial endospores (Lomstein *et al.*, 2012), protists and fungi belonging to *Eukarya* (Schippers *et al.*, 2006; Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a, 2013b). Occurrence of capsid-encoding organisms has also been confirmed (Engelhardt *et al.*, 2011). Although background molecular data on bacterial and archaeal lineages inhabiting subsurface sediment above 1000 mbsf exists (e.g., Orcutt *et al.*, 2011; Inagaki *et al.* 2006), most deep-subsurface microorganisms detected so far were refractory to cultivation (Sass and Parkes, 2011). The diversity of deeply buried microorganisms remains untapped, as subseafloor prokaryotic culturability in most studies is less than 0.1% of all microscopically detected cells (D'Hondt *et al.*, 2004). Remarkably, when wide enrichment collections targeting different physiological groups such as fermenters, sulfate-reducers and methanogens were performed using different subseafloor sediments, these often led to the isolation of the same few “generalist” bacteria (e.g., Batzke *et al.*, 2007). In most cases, the retrieved bacterial genera were adapted to a broader spectrum of environmental conditions (e.g., broad temperature range for growth) compared to their surface counterparts (Sass and Parkes, 2011). So far, within subseafloor sediments, active *Bacteria* have been identified down to 518 mbsf (Bale *et al.*, 1997), active *Archaea* down to 1626 mbsf (Roussel *et al.*, 2008), and active microeukaryotes down to 159 mbsf (Orsi *et al.*, 2013a), but we are still eager to know the depth limit of the deep subsurface biosphere. Limits to microbial habitability in subseafloor sediments are set by a variety of physical and chemical parameters like temperature, pH, pressure, salinity, porosity, availability of energy, nutrients and water, and maybe also by age. The present study site is not characterized by particularly

75 extreme conditions but stands out from sites previously examined by its depth and low  
76 porosity. The depth limit of the deep biosphere remains an important issue to place  
77 bounds on the volume of the seafloor biosphere and to guide the search for deep life  
78 capabilities/adaptation and the role of microorganisms in global nutrient cycles. We  
79 hypothesized that life could exist in even deeper sediments if pore space was sufficient.  
80 In this study, we investigated the subsurface microbial communities from a core of 1927  
81 m length collected in the Canterbury Basin (344 m water depth), off the coast of New  
82 Zealand at site U1352, which was drilled during the Integrated Ocean Drilling Program  
83 (IODP) Expedition 317 with DS *Joides Resolution*. Our purpose was to investigate  
84 vertical distribution of microbial communities, abundance and evenness of taxa above  
85 and below 1000 mbsf depth. We developed a highly stringent massive parallel tagged-  
86 amplicon sequencing of 16S-18S hypervariable regions of small-subunit (SSU) rRNA  
87 gene (Fig. S1-S2, Tables S1-S3), coupled with cell counts, real-time PCR (phylogenetic  
88 and functional genes) and cultivation approaches. This rigorous method was applied to  
89 sediment/carbonate rocks spanning epochs from the Holocene to late Eocene.

90

## 91 **Materials and methods**

### 92 *Site description and sampling*

93 Three holes (A, B and C) were drilled at Site U1352 (44°56'26.62''S; 172°1'36.30''E),  
94 reaching a total depth of 1927.5 m CSF-A, and thus spanning the Holocene to late Eocene  
95 epochs. Fluorescent microspheres were used as tracers for contamination during drilling.  
96 Sampling was processed under strict contamination controls onboard and offshore and only  
97 samples with no detectable contamination were used for this study (Fulthorpe *et al.*, 2011).  
98 Onboard, only the center parts of unconsolidated sediments and intact pieces of rocks that had

99 been exposed to UV radiation after washing were kept for microbiological analyses, as reported  
100 elsewhere (Expedition 317 Scientists, 2011). Subsamples were immediately frozen at  $-80^{\circ}\text{C}$  for  
101 onshore molecular analyses, stored at  $4^{\circ}\text{C}$  under an anaerobic gas phase for later cultivation,  
102 and stored at  $4^{\circ}\text{C}$  in a 3% NaCl/3% formalin solution for cell counting. Detailed information on  
103 sampling/subsampling of sediment, contamination controls and on depth scale terminology are  
104 provided in Supplementary Text.

105

#### 106 *Lithological, physical and geochemical data*

107 Environmental data were acquired onboard during IODP Expedition 317, as reported elsewhere  
108 (Fulthorpe *et al.*, 2011).

109

#### 110 *DNA extraction, PCR amplification and contamination controls*

111 DNA extractions were made from 16 samples collected all along the core. In order to avoid  
112 contamination, all handling was carried out in a PCR cabinet exclusively dedicated to low  
113 biomass sediment samples (PCR cabinet; Captair<sup>®</sup>Bio, Erlab), using Biopur 1.5 mL Safe-Lock  
114 micro test tubes (Eppendorf), ultrapure PCR water (Ozyme) and UV-treated ( $> 40$  min)  
115 plasticware and micropipettes. Negative controls (reaction mixture without DNA) were included  
116 in each set of PCR reactions. In addition, a negative control (e.g., negative DNA extraction) was  
117 prepared for each work stage, to ensure that no contamination with exogenous amplifiable DNA  
118 occurred during the different stages of sample treatment. The FastDNA<sup>™</sup> Spin Kit for Soil  
119 (#6560-200, MP Biomedicals<sup>®</sup>) was used to perform DNA extractions, with few modifications.  
120 Detailed information on DNA extractions and PCR amplifications are provided in  
121 Supplementary Information. Primer sequences used in this study are detailed in Table S2 and  
122 primer sets for direct and nested PCR amplifications are detailed in Fig. S2.

123

#### 124 *454-Pyrosequencing*

125 For each DNA extract, four independent 25  $\mu$ L PCR amplifications were run with fusion primer  
126 pairs specific for *Bacteria*, *Archaea* and *Eukarya*, as detailed in Table S3. PCR products were  
127 pooled two by two, so as to have two independent replicates for pyrosequencing. Potential  
128 contaminants from lab reagents were excluded through the sequencing of negative-control  
129 samples and the removal of OTUs containing sequences retrieved in negative controls. Detailed  
130 information on 454-pyrosequencing, quality filtration, trimming, clustering and taxonomic  
131 affiliation are provided in Supplementary Text.

132

### 133 *Cell counts*

134 Total prokaryotic cells were enumerated in triplicate from 13 uncontaminated sediment samples  
135 collected all along the core, using the cell extraction protocol (protocol FCM-A) described by  
136 Morono *et al.* (2011) until step 9. Then, all supernatants containing extracted cells were filtered  
137 onto 0.2  $\mu$ m filters (Anodisc, Whatman) and stained with SYBR<sup>®</sup>Green I (Invitrogen), as  
138 described elsewhere (Noble and Fuhrman, 1998). Filters were counted in epifluorescence mode,  
139 with an Olympus BX60 microscope (objective 100 $\times$ , pH3, WIB filter) (details in  
140 Supplementary Text).

141

### 142 *Real-time PCR measurements*

143 Quantifications of different lineages and diverse functional genes were performed all along the  
144 core by quantitative, real-time PCR (Q-PCR). Quantifications of *Bacteria*, *Archaea*, *Eukarya*,  
145 JS1-*Chloroflexi*, and *Geobacteriaceae* were performed using previously described Q-PCR  
146 assays based on the detection of 16S or 18S rRNA (Schippers *et al.*, 2012). These assays were  
147 carried out using TaqMan<sup>®</sup> or SYBR<sup>®</sup>Green chemistries. DNA copy numbers were also  
148 determined for the following functional genes: *mcrA* for alpha subunit of the methyl coenzyme  
149 M reductase, *dsrA* for the alpha subunit of the sulfite (bi)reductase, *aprA* for the alpha subunit

150 of the adenosine-5'-phosphosulfate reductase and *cbbL* for the large subunit of the enzyme  
151 ribulose-1.5-bisphosphate carboxylase/oxygenase (RubisCO, form I “red-like”), as described  
152 elsewhere (Schippers *et al.*, 2012).

153

#### 154 *Cultures and approaches used for their analysis*

155 A sediment slurry membrane system was used for cultivation (Ferrari *et al.*, 2008) (Fig. S8;  
156 details in Supplementary Information). Different anaerobic metabolisms found in the subsurface  
157 biosphere were targeted in culture: fermentation, sulfate-reduction and  
158 methanogenesis/acetogenesis. Media, culture conditions, viability and identification procedures  
159 of cells are described in Supplementary Text.

160

#### 161 *Statistical analyses*

162 Principal Component Analysis (PCA) was used to help in visualization of high-dimensional  
163 data. An order abundance matrix was combined with environmental parameters, using  
164 XLSTAT, to assess relationships between microbial taxa and ecological variables (Addinsoft  
165 USA, New York, USA). A second complementary approach was based on regularized canonical  
166 correlation analyses (RCCA), which were performed to highlight correlations between the order  
167 abundance matrices (X) and the environmental parameters (Y) using the R software CCA  
168 package.

169

## 170 **Results and Discussion**

### 171 *Core description*



172 The core lithology was characterized by horizontal gradual layers, from unconsolidated  
173 sediments (clay, marl) to carbonate rocks (Fig. 1). The core was composed of three  
174 lithological units (UI, UII and UIII). Unit I (0–711 m CSF-A, meters of core depth  
175 below seafloor computed by conventional method A, corresponding to mbsf; see “IODP  
176 depth scale terminology” at [www.iodp.org/program-policies/](http://www.iodp.org/program-policies/)) was predominantly  
177 characterized by a transition from mud-rich sediment to marl. Unit II (711–1853 m  
178 CSF-A) consisted of hemipelagic/pelagic sediment from calcareous sandy mud to sandy  
179 sandstone. Unit III (1853–1924 m CSF-A) was characterized by a sharp change  
180 (Marshall unconformity: ~12 Ma are missing) that occurred at 1853 m CSF-A, and was  
181 formed of hemipelagic to pelagic foraminifer-bearing nannofossil limestone of early  
182 Oligocene to late Eocene age (Fig. 1). The temperature at the bottom of the hole was  
183 estimated to be in the range 60°C–100°C on the basis of thermal conductivity  
184 measurements and geochemical results (Fulthorpe *et al.*, 2011). Below 1000 m CSF-A,  
185 sediments were replaced by consolidated sedimentary calcium carbonate rocks with  
186 porous horizons of glauconite. Porosity decreased with depth and mean pore-size was  
187 around 2–4  $\mu\text{m}$  at the hole bottom. In carbonate rocks, numerous fractures and stylolites  
188 were observed (Fig. S3). Organic carbon content was low (<0.6 wt %), with only a few  
189 samples having >1 wt % TOC (Fig. 1). The organic matter quality changed from  
190 relatively labile volatile material in the shallower sediments to more stable protokerogen  
191 with increasing depth. Methane and ethane both occurred below 11.7 and 18.2 m CSF-  
192 A and the relative ethane content increased with increasing burial depth and temperature  
193 (Fig. 1). Low but increasing concentrations of C3–C5 and occasionally C6 alkanes were  
194 also measured with depth (18). pH values were close to 7.5 and stable from the surface  
195 to 1164 m CSF-A (18). Sulfate concentration decreased gradually in the first meters of

196 the core and reached the detection limit at ~16 m CSF-A (the SMTZ: sulfate-methane  
197 transition zone, was placed between 15.2 and 16.6 m CSF-A), then it remained close to  
198 the detection limit (~0.85 mM) down to 1433 m CSF-A (Fig. 1).

199

#### 200 *Vertical distribution of cells*

201 We analyzed and compared cell abundances and cell concentrations reported for  
202 different geographic sites using a standardized procedure based on cell extraction and  
203 dissolution of silicates (Noble and Fuhrman, 1998; Kallmeyer *et al.*, 2008; Morono *et*  
204 *al.*, 2011)(Fig. 2). Mean cell numbers decreased with depth from about  $1.5 \times 10^6 \pm 4.7$   
205  $\times 10^4$  cells·cm<sup>-3</sup> (n = 8) at the surface (3.76 and 15.1 m CSF-A) to  $2.5 \times 10^4 \pm 4.9 \times 10^3$   
206 cells·cm<sup>-3</sup> (n = 7) within the deepest samples (1911 and 1922 m CSF-A). The detection  
207 limit, calculated in our conditions (Kallmeyer *et al.*, 2008), was  $2.94 \times 10^3$  cells·cm<sup>-3</sup>.  
208 The depth profile (down to 600 m CSF-A) was consistent with the general depth  
209 distribution of prokaryotic cells from other subsurface sediments (Kallmeyer *et al.*,  
210 2012).

211

#### 212 *Vertical distribution of microbial taxa*

213 It is not clear what controls abundance of *Bacteria* and *Archaea* within deep marine  
214 sediments (Schippers *et al.*, 2005; Lipp *et al.*, 2008; Schippers *et al.*, 2012). Here, a  
215 real-time PCR approach was applied to quantify representatives of the three life  
216 domains. Calculated detection limits for *Bacteria*, *Archaea* and *Eukarya* were  
217 respectively  $1.6 \times 10^4$ ,  $1.1 \times 10^3$  and  $2.9 \times 10^3$  SSU rRNA gene copies per gram of

218 sediment (wet weight). *Archaea* were the most abundant within the first meters, while  
219 *Bacteria* dominated the rest of the core (Fig. 2). Archaeal SSU rRNA gene copy  
220 numbers strongly decreased with depth (from  $1.8 \times 10^6$  to  $1 \times 10^3$  gene copies·g<sup>-1</sup>,  
221 corresponding roughly to  $1 \times 10^6$  to  $6 \times 10^2$  cells·g<sup>-1</sup>) and were no longer detectable  
222 below 650 m CSF-A. A similar depth distribution was observed for eukaryotic SSU  
223 rRNA gene copy numbers, but abundances were relatively constant with depth ( $\sim 10^4$   
224 copies·g<sup>-1</sup>). Bacterial SSU rRNA gene copy numbers were low ( $\sim 10^6$  copies·g<sup>-1</sup>  $\approx 2.5 \times$   
225  $10^5$  cells·g<sup>-1</sup>) at the surface and decreased with depth up to 1600 m CSF-A ( $8 \times 10^4$   
226 copies·g<sup>-1</sup>  $\approx 2 \times 10^4$  cells·g<sup>-1</sup>).

227 Along with these measures, deep sequencing allowed the detection limits to be lowered  
228 and masked lineages to be revealed. We pyrosequenced bacterial (V4-V5), archaeal  
229 (V1-V3) and eukaryotic (V1-V3) SSU rRNA gene amplicons from sixteen depth  
230 horizons and one negative control, pooled together in one single dataset with two PCR-  
231 replicates per sample to overcome PCR and sequencing errors (Fig. S1). Sequences  
232 were grouped into OTUs (Operational Taxonomic Units) with a 97% identity threshold.  
233 Sequence composition of the OTUs was then analyzed, and OTUs entirely composed of  
234 sequences that had appeared in a single PCR only were excluded from the diversity  
235 analyses. All the sequences kept appeared at least twice independently. Potential  
236 contaminants from lab reagents were excluded through the sequencing of negative-  
237 control samples and the removal of OTUs containing sequences retrieved in negative  
238 controls. The remaining OTUs were used to calculate non-parametric diversity indices  
239 (Fig. 3, Fig. S4-S6) and compared to the SILVA 111 database for taxonomic affiliation.  
240 Pyrosequencing results were congruent with the data discussed above. Archaeal  
241 sequences could not be amplified and sequenced for samples below 634 m CSF-A, as

242 observed with real-time PCR analyses. The non-detection of archaeal 16S rRNA genes  
243 below 650 m CSF-A using two different amplification methods suggests that *Archaea*  
244 are likely rare or absent at great depths in the Canterbury Basin. Eukaryotic sequences  
245 were detected down to 1740 m CSF-A and bacterial sequences were found up to the  
246 maximal depth of 1922 m CSF-A. The observed species richness (i.e., number of  
247 OTUs) was extremely low in comparison with other microbial habitats investigated so  
248 far, including extreme environments (e.g., Roalkvam *et al.*, 2012). Indeed, only 198, 16  
249 and 40 unique bacterial, archaeal and eukaryotic OTUs, at 3% dissimilarity level, were  
250 detected in the entire cored sequence (Fig. S4, Tables S4-S5). Chao1 estimator revealed  
251 a vertical decrease in microbial richness with increasing depth (Fig. 3). Richness  
252 estimates for *Archaea* and *Eukarya* dropped off gradually with depth and reached only 2  
253 and 4 OTUs respectively at the deepest depth for which a PCR signal was obtained.  
254 Beta diversity estimators (i.e., diversity among samples) revealed a strong  
255 differentiation between communities with depth and a strong vertical structuration (Fig.  
256 S5).

257 Archaeal diversity showed high abundances of MBG-B (Marine Benthic Group B) and  
258 MCG (Miscellaneous Crenarchaeotal Group), two archaeal groups typically found in  
259 subseafloor sediments (Lloyd *et al.*, 2013). Representatives of the as yet uncultured  
260 lineages MBG-B, MBG-E (Marine Benthic Group E) and MCG were the predominating  
261 taxa in surficial layers, while MCG was the most consistently detected archaeal lineage  
262 down to 346 m CSF-A (Fig. 2). MBG-B and MCG members are heterotrophic *Archaea*  
263 frequently found in surficial marine sediments (Biddle *et al.*, 2006; Lloyd *et al.*, 2013).  
264 *Thermococcales* dominated archaeal diversity of the sediment horizon at 634 m CSF-A.  
265 Methanogens and anaerobic methanotrophs (ANME) were not detected, in agreement

266 with the real-time PCR analysis for *mcrA*. Their absence from the dataset might be due  
267 to the intervals sampled which do not correspond to the SMTZ.

268 In *Eukarya*, few protist OTUs (Stramenopiles and uncultured *Eukaryota*) were detected  
269 down to 583 m CSF-A. Sequences affiliated with the bacterivorous protists *Bicosoecida*  
270 were detected at 346 m CSF-A, raising the question of the existence of a subsurface  
271 complex trophic web. In agreement with recently published papers (Edgcomb *et al.*,  
272 2011; Orsi *et al.*, 2013a, 2013b), fungi appeared to be the most frequently detected  
273 eukaryotes in the Canterbury Basin, with 56 to 100% of the SSU rRNA gene sequences.  
274 Different shifts between *Ascomycota* and *Basidiomycota* were observed along the core  
275 (Fig. 2). *Tremellomycetes* (order *Tremellales*), *Sordariomycetes* and *Eurotiomycetes*  
276 dominated shallow depths while *Saccharomycetes* were detected at depths between 630  
277 and 1365 m CSF-A. Deeper layers were dominated by *Wallemiomycetes*,  
278 *Microbotryomycetes* and *Tremellomycetes* (order *Filobasidiales*, not found at shallow  
279 depths). These heterotrophic fungi have been described in deep sediments of other  
280 locations (e.g., Nagano and Nagahama, 2012; Richards *et al.*, 2012) and demonstrated  
281 to be active members of microbial communities (Orsi *et al.*, 2013b). So, fungi represent  
282 an important component of sediment ecosystems through their impact on nutrient  
283 cycling and mineral weathering.

284 *Bacteria* were dominated by *Chloroflexi* and *Proteobacteria*, two heterotrophic  
285 bacterial groups well-represented in subsurface sediments (Fig. 2). They comprised 67%  
286 of the sequences and 69% of the OTUs in total. However, the abundances of the two  
287 phyla were negatively correlated. *Chloroflexi* dominated microbial communities at  
288 shallow depths (above 600 m CSF-A) and their abundances and richness decreased  
289 rapidly. Reciprocally, *Proteobacteria* were found all along the core, but their relative

290 abundance showed a sharp increase below 343 m CSF-A. Among the other lineages  
291 observed in this study, *Planctomycetes*, *Nitrospirae* and the candidate division OP9  
292 were major contributors of the amplicon pool at shallow depths. Below 600 m CSF-A,  
293 *Acidobacteria*, *Firmicutes* (a phylum containing spore-formers), and two loosely  
294 defined groups of uncultured *Bacteria* (ML635J-21 and MLE1-12) were the most  
295 consistently detected lineages. Real-time PCR quantification of the JS1-*Chloroflexi*  
296 group confirmed these results as  $\sim 10^3$  to  $10^6$  SSU rRNA gene copies·g<sup>-1</sup> were detected  
297 between the sediment surface and 1532 m CSF-A. *Deltaproteobacteria* were detected  
298 above the SMTZ and at great depths. Genes encoding a functional dissimilatory sulfite  
299 (bi)reductase (*dsrA*), a key enzyme of dissimilatory sulfate reduction frequently  
300 encountered among *Deltaproteobacteria*, was quantified above the SMTZ and in layers  
301 up to 1000 m deep in the sediment. The gene became undetectable below this depth,  
302 either because it may decrease below the detection limit or because the detected  
303 *Deltaproteobacteria* cannot respire sulfate.

304

#### 305 *Diversity and environmental factors*

306 Principal component analyses (PCA) coupled with regularized canonical correlation  
307 analyses (RCCA) were performed to visualize relationships between environmental  
308 factors and microbial taxa. We first evaluated the relationships between all  
309 environmental parameters measured (i.e., depth, pH, salinity, porosity, alkalinity and  
310 concentrations of calcium, calcium carbonate, ammonium, magnesium, sulfate,  
311 inorganic carbon, organic carbon, methane and ethane) to design a network of  
312 correlations. Only the six most explanatory variables were kept (Fig. S6). This

313 complementary analysis reinforced the conclusion about microbial distribution pattern  
314 and vertical community composition, depth being defined as a main factor explaining  
315 diversity changes (Fig. S7).

316

317

### 318 *Handling deeply buried microorganisms*

319 Cultivation approaches allowed prokaryotic and eukaryotic strains to be grown,  
320 corresponding to a fraction of the microbial communities detected all along the core,  
321 underlining that these microorganisms were viable. Fungal strains were obtained at 21  
322 to 765 m CSF-A, using elevated hydrostatic pressure to mimic *in situ* conditions (Fig. 4  
323 A-C, Table S6). Sequencing of the ITS1 rRNA regions allowed identification of a  
324 *Cadophora* representative that had already been found in extreme environments, i.e.,  
325 Antarctic environments (Tosi *et al.*, 2002) and deep-sea hydrothermal vents (Burgaud *et*  
326 *al.*, 2009) (Table S6). Fifty-seven anaerobic fungi, currently under description, have  
327 also been isolated from these sediments (Rédou and Burgaud, unpublished data). In  
328 addition to the important finding that living fungi could be cultivated from the sediment  
329 samples, microbial colonies were grown anaerobically at 60-70°C from calcareous  
330 chalk/limestone samples collected at 1827 and 1922 m CSF-A (Fig. 4 D-E), using a  
331 microcultivation method (Fig. S8). The microcolonies were successfully transferred to  
332 liquid media and subcultured. From the different tests performed, it was impossible to  
333 grow true methanogens and true sulfate-reducers. Only bacterial fermentative strains  
334 degrading the organic compounds supplied (i.e., low quantity of yeast extract) have  
335 grown. Within these subcultures, mean cell densities were low, around  $4 \times 10^5$  cells·mL<sup>-1</sup>  
336 <sup>1</sup> and growth rates were slow (in 2.5 years of culture, only 6 to 9 subcultures at 1/40 or

1/50 have been performed). Cells were able to grow at atmospheric pressure and at the estimated *in situ* pressure (22 MPa). They were composed of viable very small rods, coccobacilli and cocci of 300 to 800 nm in diameter, often forming aggregates (Fig. 4 F-I). These small sizes and this cellular organization as consortia raises questions about the living conditions of these cells and their (in)dependence with regard to other cells. The smallest diameter of a cell that assures its viability was calculated as ~ 200 nm (Velimorov, 2001). The major lineages identified in DNA and RNA libraries from these subcultures belonged to *Alpha*-, *Beta*-, *Gamma*-*proteobacteria*, *Actinobacteria* and *Armatimonadetes* (Fig. 5). With the exception of *Armatimonadetes*, all these taxa were detected from pyrosequencing in crude samples from 1827 and 1922 m CSF-A. The majority of the sequences had relatives recovered from environments with similar physical-chemical characteristics (Lin *et al.*, 2006; Mason *et al.*, 2010) (i.e., hot and reduced habitats) compared with the Canterbury subseafloor. Considering the 'ubiquity' of these taxa, one can hypothesize that they are generalist bacteria which would have been maintained during progressive burial of sediments or by transportation through circulating fluids. They might have acquired metabolic capabilities enabling them to resist the associated environmental changes. However, this hypothesis needs to be analyzed in detail. Furthermore, similar SSU rRNA gene sequences do not automatically correspond to identical physiologies, identical phenotypes or similar functions.

357

#### 358 *Impact of potential contaminants on native microbial populations*

359 Contamination is a crucial issue when working with subseafloor sediments. In general,  
360 contamination during drilling is still difficult to predict. During IODP Expedition 317,



the level of contamination during drilling was evaluated by using fluorescent microspheres and only samples with no detectable contamination were kept for microbiological analyses. Nevertheless, samples without microspheres are not necessarily uncontaminated (Smith *et al.*, 2000). Contamination generally decreases from the exterior to the interior of both sediment and rocks cores (e.g., Lever *et al.*, 2006). In consequence, only the interior of sediment cores and intact pieces of rocks that had been exposed to UV light after washing were used for the analyses. In addition, for molecular experiments deeply frozen samples of more than 1 cm in diameter were sterilized by flaming. Afterwards, all possible contaminations during the wet-lab steps have been strictly controlled and minimized (see Supplementary Text). The cutting-edge strategy applied for the pyrosequencing and bioinformatic analyses allowed removing potential spurious sequences and OTUs likely to contain contaminants, by sequencing of negative controls, a duplicate procedure and an associated bioinformatics pipeline. In addition to these precautions, the level of potential contamination of our samples was estimated by calculating the number of contaminating cells per gram of sediment and per gram of sedimentary rock based on the mean contamination values with drilling fluids and mean cell abundances in surface waters reported in the literature. The mean potential contamination was estimated as (i)  $0.011 \pm 0.018 \mu\text{L}$  of drilling fluid per gram for unconsolidated sediments drilled using advanced piston coring (APC) and (ii)  $0.027 \pm 0.029 \mu\text{L}\cdot\text{g}^{-1}$  for rocks collected using rotary core barrel (RCB) (Lever *et al.*, 2006). Considering these levels of contamination, mean cell counts of  $5 \times 10^5$  cells $\cdot\text{mL}^{-1}$  in surface waters in the ocean (Whitman *et al.*, 1998) and average densities of  $1.85 \text{ g}\cdot\text{cm}^{-3}$  in sediments and  $1.99 \text{ g}\cdot\text{cm}^{-3}$  in sedimentary rocks at site U1352, potential contamination of the interior of the core sample should be expected very low with 5 to

385 11 cells·g<sup>-1</sup> of sediment only. A second reported estimate indicates that less than 50  
386 cells per gram of sediment contaminated APC core centers drilled with *Joides*  
387 *Resolution* and that XCB cores were generally more contaminated with contamination  
388 levels 3 to 10 times higher in XCB cores than in APC core centers (House *et al.*, 2003).  
389 Considering these different estimates of potential contamination, the observed cell  
390 counts at site U1352 were 2 to 5 orders of magnitude higher in the studied samples. If  
391 contamination cannot be excluded, in the worst case, non-indigenous cells represent  
392 only up to 1% of total cells in the sample. Therefore, it is most likely that more than  
393 99% of the counted cells are native to the sampled sediment/rocks. This implies that the  
394 vast majority of the prokaryotic and eukaryotic DNA subjected to pyrosequencing was  
395 therefore derived from the sediment native cells. By extension, assuming that most of  
396 the prokaryotic DNA extracted from sediments samples is from native cells, the fact that  
397 cultivated bacteria match OTUs abundant in the crude sediment samples supports the  
398 idea that these cultivated strains are isolates of native bacteria. Consequently, the  
399 potential impact of contaminants on each category of data (cell counts, molecular data  
400 and cultures) is likely very low.

401

#### 402 *Ecological implications and future prospects*

403 We have underlined that the seafloor of the Canterbury basin hosts microorganisms  
404 which comprise *Bacteria*, *Archaea* and *Eukarya*. Some of these microorganisms are  
405 alive and, at least to a certain extent, revivable. The communities exhibit a quite low  
406 phylogenetic diversity, but this does not necessarily correspond to a low functional  
407 diversity. This poor diversity could be explained if natural selection has produced (i)  
408 taxa adapted to harsh subsurface conditions (i.e., specialists), which would be expected

409 in the case of a low connectivity among habitats; and/or (ii) taxa with a broad  
410 physiological plasticity, allowing them to survive in a diversity of nutritional and  
411 physical-chemical conditions (i.e., generalists). In fact, some taxa detected through their  
412 16S/18S rRNA gene sequences are thought to be endemic to subsurface habitats, while  
413 others seem ubiquitous and are consistently encountered in common and extreme  
414 environments. The bacterial strains in cultures are related to opportunistic or generalist  
415 taxa isolated from a broad array of redox environments, which raises the question of the  
416 existence of microbial metabolic versatility and also questions the species concept, since  
417 behind a given name or a given OTU can lay a variety of microorganisms with different  
418 ecological lifestyles. Metabolic versatility has already been demonstrated in well-known  
419 taxa. For example, some *Thermococcales* strains, which are usually fermenters that  
420 reduce sulfur compounds, can grow in oligotrophic conditions or can oxidize carbon  
421 monoxide (Sokolova *et al.*, 2004). Heterotrophy is likely to be the major mode of  
422 carbon assimilation within microbial communities of subsurface marine sediments  
423 (Batzke *et al.*, 2007). Our culture data support this hypothesis. Genome and  
424 metagenome analyses would allow functions to be predicted on a finer scale, to assess  
425 and hypothesize the individual ecological functions within the analyzed habitat or  
426 ecosystem (Vandenkoornhuyse *et al.*, 2010). The detection of fungal sequences at great  
427 depths and our success in the cultivation of fungal strains leads us to ask what role they  
428 play in deep carbon cycling and what involvement they have in dynamics/regulation of  
429 prokaryotic populations, if they are active *in situ*.

430 The broad polyphasic approach developed in this study provides direct evidence that  
431 viable microorganisms can be present in rocks that are hardened but not totally  
432 cemented, where stylolites and micro-fluid circulations exist. Our data demonstrate that

433 the combination of physical, chemical and energetic constraints encountered from 0 to  
434 1922 m CSF-A in the subseafloor of the Canterbury Basin still allow microorganisms to  
435 persist down to at least 650, 1740 and 1922 m CSF-A for *Archaea*, fungi and *Bacteria*,  
436 respectively. It extends the subseafloor sedimentary depths at which subseafloor  
437 organisms are known to be present to 1740 m for fungi and to 1922 m for *Bacteria*.  
438 Nevertheless, one cannot exclude that some of the detected sequences belong to  
439 microorganisms in dormancy. More extensive sequencing efforts will be required, i.e.,  
440 direct metatranscriptomics, to describe more directly the microbial communities along  
441 with functional signatures, and to compile data on biogeochemical processes and fluxes.

442

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453

454 Supplementary information is available at the ISMEJ's website.

455 The authors declare no conflict of interest.

## 456   **References**

- 457   Goodman K, Rochelle PA, Marchesi JR, Fry JC, Weightman AJ   et al. (1997)  
458       Desulfovibrio profundus sp. nov., a novel barophilic sulfate-reducing bacterium  
459       from deep sediment layers in the Japan Sea. *Int J Syst Bacteriol* 47:515-521.
- 460   Batzke A, Engelen B, Sass H, Cypionka H (2007) Phylogenetic and physiological  
461       diversity of cultured deep-biosphere bacteria from equatorial Pacific Ocean and  
462       Peru Margin sediments. *Geomicrobiol J* 24:261-273.
- 463   Biddle JF, Lipp JS, Lever MA, Lloyd KG, Sørensen KB, Anderson R et al. (2006)  
464       Heterotrophic Archaea dominate sedimentary subsurface ecosystems off Peru.  
465       *Proc Natl Acad Sci USA* 103:3846-3851.
- 466   Biddle JF, White JR, Teske AP, House CH (2011) Metagenomics of the subsurface  
467       Brazos-Trinity Basin (IODP site 1320): comparison with other sediment and  
468       pyrosequenced metagenomes. *The ISME J* 5:1038-1047.
- 469   Burgaud G, Le Calvez T, Arzur D, Vandenkoornhuyse P, Barbier, G (2009) Diversity  
470       of culturable marine filamentous fungi from deep-sea hydrothermal vents.  
471       *Environ Microbiol* 11:1588-1600.
- 472   D'Hondt S, Jørgensen, B B, Miller, DJ, Batzke A, Blake R, Cragg BA et al. (2004)  
473       Distributions of microbial activities in deep subseafloor sediments. *Science*  
474       306:2216-2221.
- 475   Edgcomb VP, Beaudoin D, Gast R, Biddle JF, Teske A (2011) Marine subsurface  
476       eukaryotes: the fungal majority. *Environ Microbiol* 13:172-183.
- 477   Engelhardt T, Sahlberg M, Cypionka H, Engelen B (2011) Induction of prophages from  
478       deep-subseafloor bacteria. *Environ Microbiol Rep* 3:459-465.

479 Expedition 317 Scientists (2011) Methods, *Proc. IODP 317* (Integrated Ocean Drilling  
480 Program Management International, Inc.), doi:10.2204/iodp.proc.317.102.2011.

481 Ferrari BC, Winsley T, Gillings M, Binnerup S (2008) Cultivating previously  
482 uncultured soil bacteria using a soil substrate membrane system. *Nature*  
483 *Protocols* 3:1261-1269.

484 Fulthorpe CS, Hoyanagi K, Blum P, the Expedition 317 Scientists (2011) Site U1352,  
485 *Proceedings of the Integrated Ocean Drilling Program 317* (Integrated Ocean  
486 Drilling Program Management International, Inc., Tokyo),  
487 doi:10.2204/iodp.proc.317.104.2011.

488 House CH, Cragg BA, Teske A, Leg 201 Scientific Party (2003) Drilling contamination  
489 tests during ODP Leg 201 using chemical and particulate tracers. *Proceedings of*  
490 *the Ocean Drilling Program, Initial Reports*, eds D'Hondt SL, Jørgensen BB,  
491 Miller DJ et al. (Ocean drilling Program, College Station, TX), Vol. 201, pp 1-  
492 19.

493 Inagaki F, Nunoura T, Nakagawa S, Teske A, Lever Mark, Lauer A *et al.* (2006)  
494 Biogeographical distribution and diversity of microbes in methane hydrate-  
495 bearing deep marine sediments on the Pacific Ocean Margin. *Proc Natl Acad Sci*  
496 *USA* 103:2815-2820.

497 Kallmeyer J, Smith DC, Spivack AJ, D'Hondt S (2008) New cell extraction procedure  
498 applied to deep subsurface sediments. *Limnol Oceanogr Methods* 6:236-245.

499 Kallmeyer J, Pockalny R, Adhikari RR, Smith DC, D'Hondt S (2012) Global  
500 distribution of microbial abundance and biomass in subseafloor sediment. *Proc*  
501 *Natl Acad Sci USA* 109:16213-16216.

502 Lever, MA, Alperin M, Engelen B, Inagaki F, Nakagawa S, Steinsbu BO *et al.* (2006)  
 503 Trends in basalt and sediment core contamination during IODP Expedition 301.  
 504 *Geomicrobiol J* 23:517-530.

505 Lin LH, Wang P-L, Rumble D, Lippmann-Pipke J, Boice E, Pratt LM *et al.* (2006)  
 506 Long-term sustainability of a high-energy, low-diversity crustal biome. *Science*  
 507 314:479-482.

508 Lipp JS, Morono Y, Inagaki F, Hinrichs K-U (2008) Significant contribution of Archaea  
 509 to extant biomass in marine subsurface sediments. *Nature* 454:991-994.

510 Lloyd KG, Schreiber L, Petersen DG, Kjeldsen KU, Lever MA, Steen AD *et al.* (2013)  
 511 Predominant archaea in marine sediments degrade detrital proteins. *Nature*  
 512 496:215-218.

513 Lomstein BA, Langerhuus AT, D'Hondt S, Jørgensen BB, Spivack AJ (2012)  
 514 Endospore abundance, microbial growth and necromass turnover in deep sub-  
 515 seafloor sediment. *Nature* 484:101-104.

516 Mason OU, Nakagawa T, Rosner M, Van Nostrand JD, Zhou J, Maruyama A *et al.*  
 517 (2010) First investigation of the microbiology of the deepest layer of ocean  
 518 crust. *PLoS One* 5:e15366.

519 Morono Y, Kallmeyer J, Inagaki F, the Expedition 329 Scientists (2011) Preliminary  
 520 experiment for cell count using flow cytometry. *Proceedings of the Integrated*  
 521 *Ocean Drilling Program 329* (Integrated Ocean Drilling Program Management  
 522 International, Inc., Tokyo), <http://dx.doi.org/10.2204/iodp.proc.329.110.2011>.

523 Nagano Y, Nagahama T (2012) Fungal diversity in deep-sea extreme environments.  
 524 *Fungal Ecol* 5:463-471.

525 Noble RT, Fuhrman JA (1998) Use of SYBR Green I for rapid epifluorescence counts  
526 of marine viruses and bacteria. *Aquat Microb Ecol* 14:113-118.

527 Orcutt BN, Sylvan JB, Knab NJ, Edwards KJ (2011) Microbial Ecology of the Dark  
528 Ocean above, at, and below the Seafloor. *Microbiol Mol Biol Rev* 75:361-422.

529 Orsi WD, Edgcomb VP, Christman GD, Biddle JF (2013a). Gene expression in the deep  
530 biosphere. *Nature* 499, 205–208, doi:10.1038/nature12230.

531 Orsi W, Biddle JF, Edgcomb V (2013b) Deep sequencing of subseafloor eukaryotic  
532 rRNA reveals active Fungi across marine subsurface provinces. *PLOS One*  
533 8:e56335.

534 Parkes RJ, Cragg BA, Wellsbury P (2000) Recent studies on bacterial populations and  
535 processes in subseafloor sediments: A review. *Hydrogeol Rev* 8:11-28.

536 Pawlowski J, Christen R, Lecroq B, Bachar D, Shahbazkia RH, Amaral-Zettler L *et al.*  
537 (2011) Eukaryotic Richness in the Abyss: Insights from Pyrotag Sequencing.  
538 *PLoS One* 6:e18169.

539 Richards TA, Jones MDM, Leonard G, Bass D (2012) Marine Fungi: Their Ecology and  
540 Molecular Diversity. *Ann Rev Mar Sci* 4:495-522.

541 Roalkvam I, Dahle H, Chen Y, Jørgensen SL, Haflidason H, Steen IH *et al.* (2012)  
542 Fine-Scale Community Structure Analysis of ANME in Nyegga Sediments with  
543 High and Low Methane Flux. *Front Microbiol* 3: e216.

544 Roussel EG, Cambon Bonavita M-A, Querellou Joël, Cragg BA, Webster G, Prieur D,  
545 Parkes RJ *et al.* (2008) Extending the sub-sea-floor biosphere. *Science* 320:1046.

546 Sass H, Parkes RJ (2011) Sub-seafloor Sediments: An Extreme but Globally Significant  
547 Prokaryotic Habitat (Taxonomy, Diversity, Ecology), *Extremophiles handbook*,  
548 Ed. Horikoshi K (Springer, Tokyo), pp 1016-1036.



549 Schippers A, Neretin LN, Kallmeyer J, Ferdelman TG, Cragg BA, Parkes RJ Jørgensen  
 550 BB *et al.* (2005) Prokaryotic cells of the deep sub-seafloor biosphere identified  
 551 as living bacteria. *Nature* 433:861-864.

552 Schippers A, Neretin LN (2006) Quantification of microbial communities in near-  
 553 surface and deeply buried marine sediments on the Peru continental margin  
 554 using real-time PCR. *Environ Microbiol* 8:1251-1260.

555 Schippers A, Kock D, Höft C, Köweker G, Siegert M (2012) Quantification of  
 556 microbial communities in subsurface marine sediments of the Black Sea and off  
 557 Namibia. *Front Microbiol* 3:16.

558 Smith DC, Spivack AJ, Fisk MR, Haveman SA, Staudigel H (2000) Tracer-based  
 559 estimates of drilling-induced microbial contamination of deep sea crust.  
 560 *Geomicrobiol J* 17:207-219.

561 Sokolova TG, Jeanthon C, Kostrikina NA, Chernyh NA, Lebedinsky AV, Stackebrandt E, *et al.*  
 562 (2004) The first evidence of anaerobic CO oxidation coupled with H<sub>2</sub> production by a  
 563 hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Extremophiles*  
 564 8:317–323.

565 Tosi S, Casado B, Gerdol R, Caretta, G (2002) Fungi isolated from Antarctic mosses.  
 566 *Polar Biol* 25:262-268.

567 Vandenkoornhuyse P, Dufresne A, Quaiser A, Gouesbet G, Binet F, Francez AJ *et al.*  
 568 (2010) Integration of molecular functions at the ecosystemic level:  
 569 breakthroughs and future goals of environmental genomics and post-genomics.  
 570 *Ecol Lett* 13:776–791.

571 Velimorov B (2001) Nanobacteria, Ultramicrobacteria and Starvation Forms: a search  
 572 for the smallest metabolizing bacterium. *Microb Environ* 16:67-77.

573 Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: The unseen majority. *Proc*  
574 *Natl Acad Sci USA* 95:6578-6583.

575 **Figure legends**

576

577 **Figure 1** Main physical-chemical characteristics of the studied core. Black arrows  
578 indicate measures of *in situ* temperatures used to calculate a thermal gradient of  
579 46°C/km. This thermal gradient together with the interpretation of the thermal maturity  
580 gradient defined by  $T_{\max}$  measurements, allowed estimating a bottom-hole temperature  
581 comprised between 60 and 100 °C (Source: IODP report 317) (Fulthorpe *et al.*, 2011).

582

583 **Figure 2** Lithological structure and age of lithological deposits at site U1352 in the  
584 Canterbury Basin compared with depth distribution of cell counts, 16S/18S rRNA gene-  
585 tag sequences and DNA copy numbers of genetic markers and functional genes. On the  
586 left, cell concentrations counted at site U1352 (red dots) and neighboring site U1351  
587 (blue squares), according to depth (m CSF-A), compared with the general depth  
588 distribution of cells (grey dots) in subseafloor sediments (Kallmeyer *et al.*, 2012). In the  
589 center, Phylum Class\_Order distribution of archaeal, eukaryotic and bacterial 16S/18S  
590 rRNA gene-tag sequences (based on SILVA111 classification) from OTU containing  
591 100 or more sequences (the remaining sequences were grouped into “Other”). On the  
592 right, copy numbers of the disulfite reductase genes *A*, of the 18S rRNA genes from  
593 total *Eukarya* and of 16S rRNA genes from total *Bacteria*, *Archaea* and  
594 *JS1/Chloroflexi*-related bacteria. *Geobacteriaceae* were detected only up to 15 m CSF-  
595 A ( $\sim 5 \times 10^3$  copies per g). No amplification from greater depth was shown. The  
596 functional genes *mcrA*, *aprA* and *cbbL* were not detected at all. Legend: § In other  
597 classifications, MCG affiliate with the *Thaumarchaeota*. # Lineages of plants and algae.  
598 \* Depth horizons where  $\geq 100$  reads of plants and Chrysophyceae were detected.

599

600 **Figure 3** Community richness (Chao1 non-parametric estimator) for archaeal,  
601 eukaryotic and bacterial sequences making up the OTUs (calculated by MOTHUR at  
602 3% difference between OTUs).

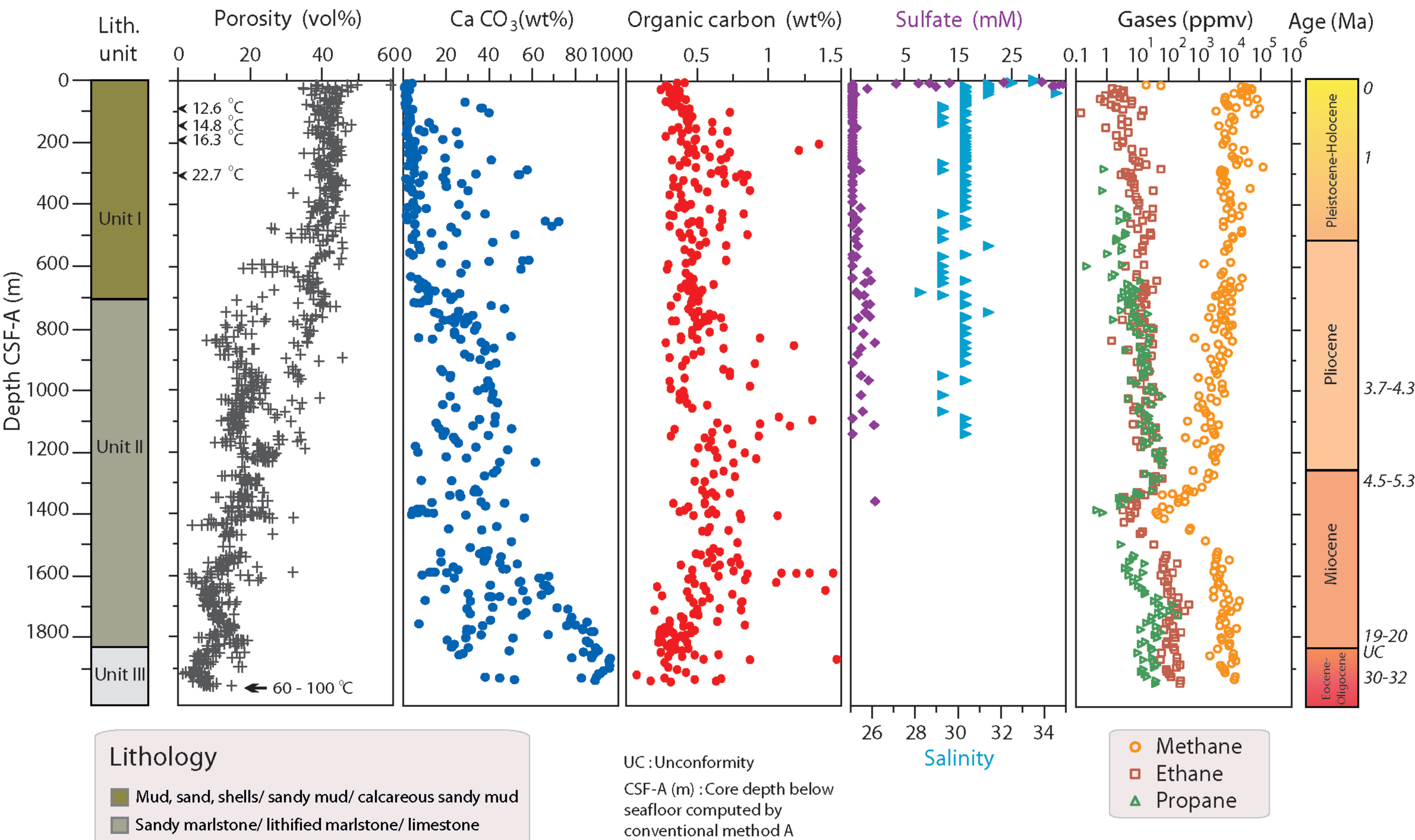
603

604 **Figure 4** Epifluorescence microphotographs of initial enrichment cultures and  
605 subsequent liquid subcultures hybridized with the Cy3-labelled probe Euk516 (staining  
606 of nuclei with DAPI) (top panel), or stained with SYBR<sup>®</sup>Green I (middle panel + G) or  
607 with the dual staining LIVE/DEAD<sup>®</sup>Bacterial Viability Kit (H-I). (A-C) Identification  
608 of fungus-like eukaryotic cells with fluorescently-labeled 18S rRNA oligonucleotide  
609 probes in initial enrichment cultures with sediments from 21 m CSF-A on PDB 3%, at 4  
610 MPa (A), with sediments from 37 m CSF-A on PDB 3%, at 11 MPa (B) and with  
611 sediments from 765 m CSF-A on PDB 0%, at 11 MPa (C). (D-E) Microcolonies  
612 observed on polycarbonate membranes (initial enrichment cultures) after 15 days of  
613 incubation with sediments from 1922 m CSF-A on H<sub>2</sub>/CO<sub>2</sub> + YE. (F-G) Cellular  
614 aggregates observed in the 7th liquid subcultures performed after the initial enrichment  
615 with sediments from 1827 m CSF-A on YE + peptone + casamino acids. (H-I) Cell  
616 structural integrity was observed in the 7<sup>th</sup> liquid subcultures performed after the initial  
617 enrichment with sediments from 1827 m CSF-A on acetate + YE (H) and with  
618 sediments from 1922 m CSF-A on YE + peptone + casamino acids (I). Legend: YE,  
619 Yeast Extract; PDB, Potato Dextrose Broth.

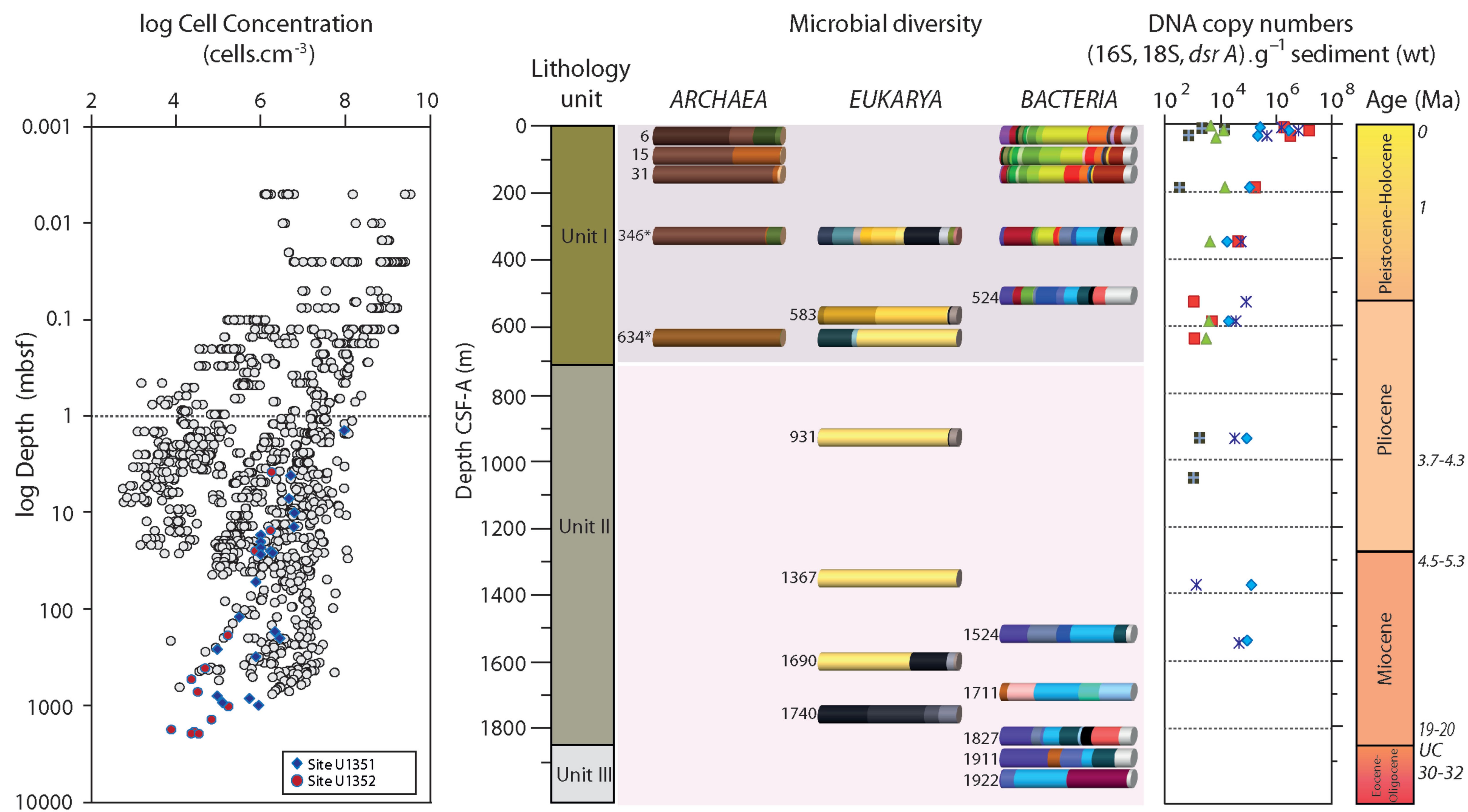
620

621 **Figure 5** Phylogenetic position of the bacterial 16S rRNA gene sequences detected in  
622 RNA extracts from the fifth to eighth liquid subculture following the initial enrichment  
623 step on a sediment substrate membrane system. The phylogenetic reconstruction was  
624 performed using the Neighbor-Joining algorithm. Bootstrap values based on 1000  
625 replications are indicated at the branch nodes. Representative bacterial 16S rRNA gene  
626 sequences of H<sub>2</sub>/CO<sub>2</sub> + yeast extract (YE)-based cultures and acetate + YE-based  
627 cultures from the 137R section (1827 m CSF-A), as well as YE + peptone (pept.) +  
628 casamino acids (CA)-based culture from the 148R section (1922 m CSF-A) are shown  
629 in bold blue letters. Sequences from basalt or an underground aquifer where the  
630 temperature is high (52<T°<80°C) are shown in brown; Sequences from environments  
631 rich in hydrocarbons or radioactive metals are shown in orange. Accession numbers are  
632 given in brackets.









### Archaea

#### Crenarchaeota

- MBG - B
- MCG s
- Unclassified Crenarchaeota

#### Euryarchaeota

- Thermococcales
- MBG - E
- SAGMEG
- Other

### Bacteria

- Acidobacteria\_Acidobacteria\_DA052
- Acidobacteria\_Acidobacteria\_S035
- Actinobacteria\_Actinobacteria\_Corynebacteriales
- Actinobacteria\_OPB41
- Firmicutes\_Bacilli\_Bacillales
- Nitrospirae\_Nitrospira\_Nitrospirales
- Planctomycetes\_Phycisphaerae\_MSBL9
- Spirochaetes\_Spirochaetes\_Kazan-3B-09
- BHI80-139
- Candidate\_division\_OP9
- Uncultured Bacteria\_ML635J-21
- Uncultured Bacteria\_MLE1-12
- Other

### Eukarya

#### Streptophyta #

- Liliopsida\_Poales
- Eudicotyledons\_Malpighiales
- Eudicotyledons\_Rosales
- Eudicotyledons\_Solanales

#### Stramenopiles

- Bicosoecida\_Bicosoecida
- Chrysophyceae\_
- Ochromonadales #

#### Ascomycota

- Dothideomycetes\_Capnoidiales
- Eurotiomycetes\_Chaetothyriales
- Eurotiomycetes\_Mycocaliciales
- Unclassified\_Sordariomycetes
- Saccharomycetes\_Saccharomycetales

- Uncultured\_Eukaryota
- Other

#### Chloroflexi

- Caldilineae\_Caldilineales
- GIF3
- GIF9
- JG30-KF-CM66
- MSB-5B2
- MSBL5
- Napoli4B-65
- Sh765B-AG-111
- vadinBA26

#### Proteobacteria

- Alphaproteobacteria\_Caulobacteriales
- Alphaproteobacteria\_Rhizobiales
- Alphaproteobacteria\_Sphingomonadales
- Betaproteobacteria\_Burkholderiales
- Betaproteobacteria\_Neisseriales
- Deltaproteobacteria\_Desulfarculales
- Deltaproteobacteria\_Desulfobacteriales
- Deltaproteobacteria\_Myxococcales
- Gammaproteobacteria\_Pseudomonadales
- Gammaproteobacteria\_Xanthomonadales

### Real time-PCR

- Bacteria
- Archaea
- Eukarya
- JS1/Chloroflexi
- dsrA*

### Lithology

- Mud, sand, shells/  
sandy mud/  
calcareous sandy mud
- Sandy marlstone/  
lithified marlstone/  
limestone
- Nannofossil limestone

UC : Unconformity

CSF-A (m) : Core depth below  
seafloor computed by  
conventional method A



